FIXATION OF "NEUTRALIZED" INFLUENZA VIRUS BY SUSCEPTIBLE CELLS[1]

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It is well known that specific antibody, under appropriate conditions, will prevent viral infections, but thorough knowledge concerning how this is accomplished is incomplete. Andrewes (1929) after working with virus III in tissue cultures suggested the possibility that immune serum acts by preventing the virus from entering the cells; if the serum fails to do this, invasion of cells occurs. Sabin (1935) working with vaccinia, pseudorabies, and herpes viruses concluded that a union does not take place between these viruses and the protective substance in antiviral sera. He believed that if a virus was fixed first to the susceptible cell, infection took place, whereas if the protective substance was fixed first or simultaneously with the virus, infection did not occur.

Adsorption studies have furnished convincing evidence that influenza virus forms a sufficiently strong union with the antibody and that the antibody along with the virus is removed by centrifugation (Smorodintseff *et al.*, 1936; Burnet, 1937; Smith and Andrewes, 1938; Friedewald, 1944). The influenza virus-antibody complex would seem to be a satisfactory one to use in determining whether or not this neutralized virus could make contact with the susceptible cell. Neutralized virus is not always easy to detect, but the reactivation method of McKee and Hale (1948), using homologous concentrated inactive virus, seemed to be applicable to this particular problem. Although a number of different cells are known to fix influenza virus, those lining the allantoic sac of the embryonated chicken egg were chosen for the initial investigation. This study was undertaken to determine whether neutralized influenza virus is fixed to these susceptible cells.

MATERIALS AND METHODS

Active influenza virus. The type A strain of virus used was isolated in Iowa City during the 1943 epidemic. Fresh viral suspensions were obtained by inoculating 0.1 ml of a 10^{-3} dilution of the seed virus into the allantoic sac of 10-day old embryonated hen's eggs and incubating them at 37 C. Twelve and eight-tenths units, *vide infra*, of influenza antiserum, type B (Lee), were added to the seed virus suspension prior to inoculation to obviate the possibility of type B contamination. The allantoic fluid was harvested 48 hours later. No fluid that had been harvested for more than 2 hours was used in the experiments.

The freshly harvested allantoic fluid had a titer of 1:9,600 (constituting 9,600 viral units per ml) as determined by the Salk modification of the Hirst hemagglutination technic (Salk, 1944). The egg infectivity titer was 10^{-9}. All seed virus was stored at 4 C. Penicillin and streptomycin were added to all inocula for embryos to prevent bacterial growth; 1,000 units of each per ml of inoculum were used throughout this study. The antibiotics were included and figured in the dilutions used.

Concentrated heat inactivated influenza virus. Inactivated virus was prepared by concentrating freshly harvested allantoic fluid by evaporation until it had a hemagglutinin titer of 1:76,800 to 1:102,400. To accomplish this the allantoic fluid was centrifuged to remove tissue debris and then placed in a sterile cellophane dialyzing tube. The suspension was allowed to dialyze at 4 C in approximately 100 volumes of double-distilled water for 24 hours to remove the dialyzable salts; if the salts are not removed prior to evaporation, a voluminous precipitation occurs. The cellophane tube was placed in a specially designed device[2] to concentrate the suspension 10 times, after which the concentrate was heated to 57 C for 80 minutes. The hemagglutinin titer fell to 1:25,600 and the material failed to produce hemagglutinin when 0.1 ml of a 10^{-0} and a 10^{-3} suspension was inoculated into the allantoic sac and incubated for 48 hours. After two subsequent embryo passages the product still failed to produce hemagglutinin.

Influenza antiserum type IA43. The antiserum was developed in four week old albino Swiss mice from an inbred strain at the State University of Iowa. The antigen was from mice that received type B antiserum along with the virus. Each animal used for antiserum preparation received six intraperitoneal injections of live virus five days apart. The first dose consisted of 0.1 ml of a 10^{-2} mouse lung suspension; the second, 0.1 ml of a 10^{-1} suspension; the third, 0.2 ml of a 10^{-1} suspension; the fourth, 0.3 ml of a 10^{-1} suspension; the fifth, 0.4 ml of a 10^{-1} suspension; the sixth, 0.5 ml of a 10^{-1} suspension. Ten days were allowed to pass after the last injection before the mice were bled from the heart. The serum was removed from the clot and stored at 20 C. The antiserum had an antihemagglutination titer of 1:38,400 (constituting 38,400 units of antibody per ml) against a final dilution of four agglutinating doses of homologous virus.

Neutralization. Equal volumes of the virus and antibody dilutions required were mixed, and the mixtures were allowed to stand at room temperature for one hour during which time they were gently shaken several times. One-tenth ml of each mixture was injected into the allantoic sac of each of five 7-day old embryos. The eggs were incubated at 37 C.

Testing for neutrality. From each embryo in a test group, 0.1 ml of the allantoic fluid was harvested at 24-hour intervals, beginning after 24 hours. The harvest was discontinued for a particular test group when the pooled allantoic fluids

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[2] The device will be described in detail along with diagrams in a separate note submitted subsequently. Briefly it consists of a thermostatically controlled heating element, a motor driven fan, and a counter balanced carriage for the cellophane bag containing the infected fluid. The heating element and fan shut off when the desired reduction in weight of the fluid is reached.

from this group on two subsequent harvests proved to contain hemagglutinin or when no acceptable fluid could be harvested due to the growth of the embryo. The latter complication usually occurred 8 to 10 days after the start of the experiment.

Reactivation. One-tenth ml of the concentrated heat inactivated influenza virus was inoculated into the allantoic sac of those embryos previously injected with the neutral mixtures. The time intervals between these two injections differed with each group as indicated in the experiment. In each experiment additional embryos received 0.1 ml of normal saline, which was used in place of the concentrated heat-inactivated influenza virus to serve as control. All embryos were sampled at 24-hour intervals as when testing for neutrality. The embryos were studied until they became too large unless the experimental group showed hemagglutinin on two successive harvests before this time.

Viability determined in cellophane bags. A bag holding about 2 ml was constructed from cellophane dialyzing tubing. The diameter was 6 mm and the thickness of the wall was 0.18 mm. One end was closed by a knot and sealed with beeswax.

The bag was filled with 0.5 ml of a 10 times overneutralized viral suspension, the concentration of which equalled that of the overneutralized viral suspension used in reactivation experiments after the injection and subsequent dilution of these suspensions in the allantoic fluid. The dilution of the neutral mixture was made in normal allantoic fluid from 7-day old embryos. The dialyzing bag was then introduced into the allantoic sac through a hole made in the chorioallantoic membrane. The other end of the dialyzing bag was sealed by wax and fastened on the outside of the egg-shell. All material used was bacteriologically sterile. Fifty units each of penicillin and streptomycin per ml were included in the overneutralized virus suspension. One thousand units of each antibiotic were injected into the allantoic sac before introducing the dialyzing bag. Twenty eggs were used in each experiment, and they were incubated at 37 C. Five bags were harvested on each of the following 4 days. The bag was emptied with a syringe and washed with 0.5 ml normal saline, the washing was then added to the first contents of the bag. To this volume (usually 0.8 ml) 0.05 ml of a 10^{-1} suspension of concentrated heat inactivated virus was added for reactivation. The previously mentioned procedure was used for the detection of active virus.

Although twenty eggs were used in each of three experiments, only in a single instance was one embryo alive on the third day. One group of bags was tested on the fifth day even though the embryos in this group had been dead since the second day. Death of the embryos may have been due to the penetration of the yolk sac at an early stage of the experiment.

Fixation experiments. A 10 times overneutralized viral suspension (4.8 or 9.6 units of active virus and 200 or 400 units of homologous antibody, respectively) was made, and 0.1 ml was injected into the allantoic sac of 7-day old embryos, 5 eggs in each of three groups. After intervals of 1 hour, 12 hours, and 20 to 26 hours one group of eggs was removed from the incubator. The allantoic fluid from these eggs was pooled and ground with sterile powdered pyrex glass.

The chorioallantoic membranes were washed in 5 to 12 changes of normal sterile saline and then ground with sterile powdered pyrex glass to a homogenous suspension.

After centrifugation of the fluid and suspension of membranes for 5 minutes at 1,000 rpm, 0.05 ml of concentrated heat inactivated homologous virus was added to each ml of the preparations. This amount of inactive virus was calculated to cause no more than a minimal amount of interference to the eventually active virus present (Isaacs and Edney, 1950). After two hours' incubation at room temperature during which time the mixtures were gently agitated several times, 0.1 ml portions of the suspensions were injected into the allantoic sac of 10-day old embryos, using five eggs for each suspension. In the experiments designed to determine the amount of virus fixed by the cells of the membrane, the allantoic fluids and chorioallantoic membrane suspensions were serially diluted separately by tens before inoculation. Following incubation at 37 C for 48 hours the allantoic fluids of embryos belonging to the same groups were pooled and tested for hemagglutinin.

TABLE 1

Determination of a neutral mixture of virus and antibody in the chick embryo

UNITS		DAYS AFTER INJECTION OF NEUTRALIZED VIRUS										COMMENTS
Active virus	Antibody	1	2	3	4	5	6	7	8	9	10	
192	200	−	−	+*	+							Not neutral
96	200	−	−	+	+							Not neutral
48	200	−	−	−	−	−	−	−	−			Neutral
24	200	−	−	−	−	−	−	−	−			Neutral
12	200	−	−	−	−	−	−	−	−	−	−	Neutral

* + denotes appearance of hemagglutinin in the allantoic fluid.

To determine the effect of overneutralization on fixation the previous procedure was followed with one exception. The concentration of antibody was varied as indicated in the protocol, and the viral concentration was held constant.

EXPERIMENTAL RESULTS

To study the activities of overneutralized mixtures of virus and antibody, a neutral mixture must be determined that will suffice according to the conditions of the experiments. Active virus was serially diluted by twofold steps and mixed with specific antiserum to give viral units from 12 to 192, and the antibody concentration was kept constant at 200 units. These units are synonymous with hemagglutination and antihemagglutination units, respectively. The mixtures were incubated at room temperature for one hour and were then inoculated into embryos. At 24-hour intervals 0.1 ml samples of allantoic fluid were harvested. Like samples were pooled and assayed for hemagglutinin. The results of this experiment may be seen in table 1. It will be noted that a final concentration of 192 and 96 units of active virus was delayed but not blocked in the production

of hemagglutinins, whereas 48 or less viral units were neutralized for as long as was practical to follow the experiment.

To be certain that the overneutralized virus could be reactivated and to ascertain the influence of time upon the reactivation process, experiments were performed with these factors in mind. To avoid the equivocation that might result from the use of a "just neutral" mixture, mixtures overneutralized from 10 to 100,000 times by multiples of 10 were employed. The overneutralized mixtures were injected into the allantoic sac of the embryos, and 5 seconds later concentrated heat inactivated influenza virus was injected into the experimental group and saline into the control group. Other experimental embryos received the concentrated inactive virus at 24 and 48-hour intervals after the overneutralized mixture had been injected. The control embryos received saline in each instance. Samples of allantoic fluid were withdrawn daily until a positive hemagglutination test was obtained on two successive days, or until it was impossible to carry the experiment further.

The results are listed in table 2, and it can be seen that reactivation occurs after various incubation periods if concentrated inactive virus is added 5 seconds after the neutralized virus. However, if it is added 24 or 48 hours after the neutral mixture, no reactivation occurs.

To define more specifically the time elapsing before the addition of concentrated heat inactivated influenza virus beyond which no reactivation could occur seemed indicated. Intervals varying from 5 seconds to 24 hours were allowed to elapse between the introduction of the neutral mixture and the injection of the inactive virus. The virus-antibody ratio was kept constant throughout this experiment. The exact intervals employed and the results obtained are shown in table 3. These data show that the threshold time after which reactivation did not occur appeared to be about 24 hours.

Why should time be a limiting factor where reactivation is concerned? Does the virus perish during this 24-hour period? An attempt to answer these questions is set forth in the experiment that follows. Active virus and specific antibody mixtures were diluted in normal allantoic fluid until their respective concentrations were the same as obtained in previous experiments due to injecting them into the allantoic sac. The mixture was placed in cellophane bags which were sealed in the allantoic sacs of the embryos and allowed to remain for four days at 37 C. Each day bags were removed from some of the embryos, their contents mixed with concentrated heat-inactivated influenza virus, and injected into the allantoic sacs of new embryos to determine whether the virus had survived. The virus was observed to be active for three days using this technic. Actually, live virus was recovered from some sacs on the fifth day, but the supporting embryos were dead. This would lead one to question any suggestion that the virus was not reactivated in the previous experiments because it had become permanently inactivated. If the virus is alive after 24 hours, but cannot be reactivated, where is it? Two possible answers to this question might be: (1) it is in an area other than those being tested, or (2) it now exists in a new form that is undetectable by conventional procedures. With the O-D transformation of

TABLE 2

Reactivation of mixtures of virus and antibody 10 to 100,000 times overneutralized

UNITS		DAYS AFTER INJECTION OF OVERNEUTRALIZED MIXTURE										COMMENTS
Active virus	Antibody	1	2	3	4	5	6	7	8	9	10	
		Concentrated heat inactivated virus injected after 5 seconds										
4.8	200	+*	+									Reactivated
0.48	200	−	+	+								Reactivated
0.048	200	−	−	−	−	−	−	+	+			Reactivated
0.0048	200	−	−	−	−	−	−	−	−	−		Not reactivated
0.00048	200	−	−	−	−	−	−	−	−	−	−	Not reactivated
		Normal saline injected after 5 seconds										
4.8	200	−	−	−	−	−	−	−	−	−		Neutral
0.48	200	−	−	−	−	−	−	−	−			Neutral
0.048	200	−	−	−	−	−	−	−	−	−	−	Neutral
0.0048	200	−	−	−	−	−	−	−	−	−	−	Neutral
0.00048	200	−	−	−	−	−	−	−	−	−	−	Neutral
		Concentrated heat inactivated virus injected after 24 hours										
4.8	200	−	−	−	−	−	−	−	−	−	−	Not reactivated
0.48	200	−	−	−	−	−	−	−	−	−	−	Not reactivated
		Normal saline injected after 24 hours										
4.8	200	−	−	−	−	−	−	−	−	−	−	Neutral
0.48	200	−	−	−	−	−	−	−	−	−	−	Neutral
		Concentrated heat inactivated virus injected after 48 hours										
4.8	200	−	−	−	−	−	−	−	−	−	−	Not reactivated
0.48	200	−	−	−	−	−	−	−	−	−	−	Not reactivated

* + denotes appearance of hemagglutinin in the allantoic fluid.

TABLE 3

Reactivation of a mixture of virus and antibody 10 times overneutralized

UNITS		DAYS AFTER INJECTION OF OVERNEUTRALIZED VIRUS										CONCENTRATED HEAT INACTIVATED VIRUS INJECTED AFTER:	COMMENTS
Active virus	Antibody	1	2	3	4	5	6	7	8	9	10		
4.8	200	+*	+									5 seconds	Reactivated
4.8	200	+	+									1 hour	Reactivated
4.8	200	−	+	+								13 hours	Reactivated
4.8	200	−	+	+								20 hours	Reactivated
4.8	200	−	−	−	−	−	−	−	−	−	−	24 hours	Not reactivated

* + denotes appearance of hemagglutinin in the allantoic fluid.

influenza virus (Burnet and Bull, 1943) in mind, new attempts to reactivate the virus beyond 24 hours were made. This was done by using the guinea pig red

blood cell agglutination test instead of the more conventional chicken cell agglutination technic to check for the virus. Burnet showed that freshly isolated virus failed to agglutinate chicken cells, whereas it would guinea pig cells. The use of guinea pig cells, however, revealed no such virus present after the 24-hour period. It would be unconventional to assume that an overneutralized virus could be affixed to the cell lining of the allantoic sac because antibody should prevent this. Nevertheless, it was thought that such a possibility should be checked. Preliminary experiments indicated that some of the neutralized virus was on or in the membrane. This was the case even after the membrane had been washed in sterile saline 10 to 12 times to remove any contaminating virus from the allantoic fluid.

The possibility that overneutralized virus gradually moved from the allantoic fluid onto or into the allantoic membrane did become a real one. An experiment was designed to determine this movement. Four and eight-tenths viral units were overneutralized 10 times by 200 units of antibody, and 15 embryos were inoculated in the allantoic sac with 0.1 ml per embryo. After each of the arbitrarily-

TABLE 4

Reactivation of 10 times overneutralized virus from chorioallantoic membrane and allantoic fluid

UNITS			HOURS AFTER INJECTION OF OVERNEUTRALIZED VIRUS		
Active virus	Antibody		1	12	20
4.8	200	Membrane	+*	+	+
		Fluid	+	+	+

* + denotes hemagglutination by allantoic fluids from test embryos in a dilution of 1:20.

established time intervals (1, 12, and 20 hours) had elapsed, 5 embryos were examined. The allantoic fluids in each group were pooled and the chorioallantoic membranes washed in 5 changes of physiological salt solution. The pooled membranes were ground in a mortar with the aid of sterile powdered pyrex glass. After grinding, the membrane pulp was centrifuged and the supernatant fluid poured off and mixed with the inactive concentrated virus. After an incubation period of 2 hours at room temperature, this mixture was injected into 5 embryos. The harvested allantoic fluid was treated in the same way including grinding with pyrex glass to control any adsorption of virus. The results of this experiment are given in table 4.

It seems improbable that the virus reactivated from the membrane was merely contaminating virus not washed away, but it was impossible to rule this out. A second series of attempts to reproduce these experiments failed to show any reactivation. This led to the conclusion that perhaps too small an amount of active virus was being used in the experiment. It was felt that the virus escaped detection from time to time because it was so near the threshold amount required to infect. Accordingly, the amount of active virus and the amount of antibody were both doubled and the experiment repeated.

Two new factors were introduced in this experiment. After the preparation of the membranes and fluids they were diluted serially by tens from undiluted to 1:100. The membranes were washed twelve times instead of five. Five embryos were inoculated with each dilution of each preparation. The data in table 5 show that the increase in available active virus influenced the results.

The membrane appears to be nearly free from the neutralized virus during the first hour. Conversely, the allantoic fluid possesses enough virus so that after reactivation it can be diluted 1:10 and still infect embryos. After 12 hours in the embryo the picture is similar but the allantoic fluid is infective after reactivation only if used undiluted. The samples taken 26 hours after the embryo inoculation show a more pronounced difference; the membranes are infective after reactivation in a dilution of 1:100, whereas the allantoic fluid remains infective only if used undiluted. While one cannot compare the membrane and allantoic fluid with extreme definition, it still seems apparent that the overneu-

TABLE 5

Reactivation of 10 times overneutralized virus from chorioallantoic membrane and allantoic fluid

UNITS			HOURS AFTER INJECTION OF OVERNEUTRALIZED VIRUS		
Active virus	Antibody		1	12	26
9.6	400	Membrane undiluted	+*	++	++++
		Membrane 10^{-1}	−	−	++++
		Membrane 10^{-2}	−	−	++++
		Fluid undiluted	++(±)	++++	+++
		Fluid 10^{-1}	++++	−	−
		Fluid 10^{-2}	−	−	−

* + denotes hemagglutination by allantoic fluids from test embryos: + = 1:2 dilution of fluid causing hemagglutination; ++ = 1:20; +++ = 1:200; ++++ = 1:2,000.

tralized virus moved in a direction from the allantoic fluid to the allantoic membrane. The allantoic fluids harvested from the embryos used to detect reactivated virus were tested against guinea pig as well as chicken cells and comparable titers were obtained.

The results of this last experiment have been successfully confirmed. Though slightly less pronounced they have, nevertheless, followed the same pattern. Attempts to cause the neutralized virus to move entirely from the allantoic fluid to the membranes have been unsuccessful. Even after 102 hours a small amount appears to remain in the fluid. At no time has there appeared evidence of viral propagation prior to reactivation. Exposing the allantoic fluid and chorioallantoic membranes to the same treatment outlined before but omitting the addition of concentrated inactive virus resulted in no evidence of virus growth.

The influence of time elapsing after the inoculation of the embryos with overneutralized virus on the movement of such virus from the allantoic fluid was apparent. It seemed important also to learn the influence of the degree of

overneutralization, that is of excess antibody, on this same function. A constant amount of virus, 9.6 units, was mixed with varying concentrations of antibody to overneutralize the virus 3, 10, 50, 100, and 360 times. Each mixture was inoculated into embryos as described under methods and after 26 to 28 hours the allantoic fluids and membranes were harvested. They were treated in the usual manner, diluted, and tested for reactivation by the inoculation of new embryos. The results of this experiment, as listed in table 6, show quite clearly the influence of antibody concentration on the movement of the neutralized virus toward the membrane. The movement of the virus from the fluid towards the membrane seems inversely proportional to the excess of antibody though this does not appear to be exactly a straight line function.

TABLE 6

Reactivation of virus overneutralized 3, 10, 50, 100, and 360 times from chorioallantoic membrane and allantoic fluid

HOURS AFTER INJECTION OF OVERNEU- TRALIZED VIRUS*		DEGREES OF OVERNEUTRALIZATION				
		3	10	50	100	360
	Membrane undiluted	++++†	++	++	++	+
	Membrane 10^{-1}	++++	−	−	−	−
26–28	Membrane 10^{-2}	++++	−	−	−	−
	Fluid undiluted	++	++	+	+	±
	Fluid 10^{-1}	−	−	−	−	−
	Fluid 10^{-2}	−	−	−	−	−

* Active virus units constant at 9.6.

† + denotes hemagglutination by allantoic fluids from test embryos: + = 1:2 dilution of fluid causing hemagglutination; ++ = 1:20; +++ = 1:200; ++++ = 1:2,000.

DISCUSSION

The evidence obtained in these studies leads one to believe that influenza virus sufficiently neutralized to prevent the production of hemagglutinin can fix to a susceptible cell. The findings suggest the process to be a rather firm fixation rather than a subtle adsorption. After 12 separate washings of the membrane in saline the amount of virus retained is considerable. In addition to this the gradual disappearance of the virus from the allantoic fluid and the corresponding fixation to the susceptible cell suggest that a dynamic process is at work.

Whether or not the neutralized virus becomes fixed to the cells by the process of "viropexis" (Fazekas de St. Groth, 1948) remains unknown. Perhaps the same or a similar method is involved. The failure of a small amount of the neutralized virus to become fixed to the cells is not at odds with the findings in which virus free from antibody is employed (Henle, 1949). *In vitro* experiments on reactivation of overneutralized virus succeed in reactivating only about 10 per cent of the available virus (McKee and Hale, 1948). At the present time it is unknown whether the virus fixed to the membrane presents any unusual problems along

this line. Suffice it to say reactivation does occur, but the relative efficiency of the method as applied to this case remains to be determined.

It is suggested that influenza virus neutralization may occur in three zones; first, a zone in which insufficient antibody is present to prevent propagation or at least the production of hemagglutinin; a second zone where production of hemagglutinin is prevented but fixation of the virus to the susceptible cell is permitted. A third zone probably exists in which fixation also is prevented. Certainly the last experiments in this series indicate that fixation becomes minimal as the antibody becomes more excessive.

Any attempt to explain the significance of these findings in the establishment of infection would be premature. One cannot help but wonder, however, for what length of time a "neutralized" virus might remain fixed to a susceptible cell. Other questions that could be raised are: Is the virus any better situated to survive in close proximity to the cell, even if it cannot multiply, than it would be if fixation were prevented? Does there exist any system in the body for upsetting the equilibrium between virus and antibody that would permit the fixed virus, in time, to proceed with the infection? The answers to these questions would be interesting but difficult to obtain.

A more precise appraisal of the fixation process involving chicken red blood cells is under study.

SUMMARY

A mixture of influenza virus and excess homologous antibody injected into the chicken embryo was reactivated by the subsequent injection of homologous concentrated inactive virus.

The length of time elapsing between the injection of the neutralized virus and the introduction of inactive virus which could bring about reactivation was determined.

Virus neutralized in excess of what was required to prevent hemagglutinin production was found to fix to the cells of the chorioallantoic membrane.

The influence of time and the degree of neutralization on such fixation were studied.

Some of the theoretical implications of the findings are discussed.

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